CO-OXIDATIVE METABOLISM OF 4-AMINOBIPHENYL BY LIPOXYGENASE FROM SOYBEAN AND HUMAN TERM PLACENTA

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ABSTRACT:
4-aminobiphenyl (4-ABP) co-oxidation catalyzed by the human term placental lipoxygenase (HTPLO), purified by affinity chromatography, was studied in the presence of linoleic acid (LA). Soybean lipoxygenase (SLO) which is extensively employed as a model lipoxygenase, was used for comparison. Spectral analyses of reaction media containing 4-ABP, LA, and SLO/HTPLO suggested the disappearance of substrate (∆A at 270 nm) and a gradual appearance of a new peak at 315 nm, indicating a metabolite formation. Under optimal assay conditions, SLO exhibited a specific activity of 350 nmoles of 4-ABP depleted/min/mg of enzyme. To observe the maximal rate of co-oxidation by the HTPLO (45 nmoles of 4-ABP depleted/min/mg protein), an incubation of 50 μM 4-ABP, 2 mM LA, and 80 μg/ml protein at pH 7.4 was essential. 4-ABP was also oxidized by SLO in the presence of H2O2, although at a lower rate. The reversed-phase HPLC analysis of organic extracts of the incubations of 4-ABP with SLO and H2O2/LA as well as HTPLO and LA showed the formation of a major peak which was identified by GC-MS as 4,4′-azobis(biphenyl). The addition of GSH, BHT, and BHA to the enzymatic incubations decreased the formation of 4-ABP metabolite, suggesting the generation of a free radical as the initial metabolite during 4-ABP oxidation. Both the SLO and HTPLO mediated reactions were significantly inhibited by nordihydroguaiaretic acid, gossypol, and 5,8,11-eicosatriynoic acid. Collectively, these results suggest that the co-oxidation catalyzed by HTPLO may be the underlying biochemical mechanism responsible for the transplacental toxicity of 4-ABP.

Occupational exposure to arylamine such as 4-aminobiphenyl (4-ABP) is known to be associated with an elevated risk of bladder cancer (1). Patrianakos and Hoffmann (2) and Hammond et al. (3) have documented the presence of 4-ABP in environmental tobacco smoke. Interestingly, the concentration of 4-ABP is 30 times higher in the sidestream smoke than in the mainstream smoke. Therefore, passive smokers are likely to be exposed to a high concentration of 4-ABP. An increased risk for hematopoietic malignancies (4) and lung cancer (5) in adults was reported to be related to the gestational smoking. It has been demonstrated that carcinogens present in tobacco smoke can cross the placental barrier in man (6). Sandler et al. (4) suggested that an exposure to tobacco smoke in utero may increase the risk of developing childhood cancers. Another epidemiological study (7) has documented a strong association between the incidence of childhood and adult cancer and the risk of developing childhood cancers. Another epidemiological study (7) has documented a strong association between the incidence of childhood and adult cancer and in utero exposure to tobacco smoke carcinogens. The authors of this study observed that a dose-response relationship exists between the number of cigarettes smoked during pregnancy and cancer risk in offspring. Several in vivo studies have identified DNA (8) and hemoglobin (9) adducts of 4-ABP or its metabolites in the human placenta and cord blood. In mice, the transplacental transfer of 4-ABP was detected using 32P-postlabeling assay (10).

The oxidative metabolism of 4-ABP is essential to exert its carcinogenic effect. It has been suggested that 4-ABP-DNA/hemoglobin adduct formation is a reliable index of in vitro or in vivo N-oxidation of 4-ABP by mammalian hepatic cytochrome P-450 (11, 12). 4-ABP also undergoes bioactivation during prostaglandin synthase mediated metabolism (13). In addition, a report describing peroxidative metabolism of 4-ABP catalyzed by horseradish peroxidase (HRP), a model peroxidase enzyme system, is also available (14).

At present, information is lacking regarding the enzyme(s) responsible for 4-ABP metabolism in the human feto-placental unit. While fairly high levels of cytochrome P-450, flavin-containing monoxygenase and/or prostaglandin synthase, capable of xenobiotic oxidation exist in different human tissues, their presence is not detectable in a biologically significant level in the term placentas of nonsmokers (15–17). In a recent study, Hakkola et al. (18) examined the expression of individual xenobiotic metabolizing P-450 genes in human placenta at mRNA level by reversed transcriptase-polymerase chain reaction (RT-PCR). mRNAs of several CYP forms were detected at a very low level. Except for CYP1A1, a cigarette smoke-induced form, these forms could not be detected at protein levels. These reports have triggered interest in the exploration of alternative enzymatic pathway(s) of 4-ABP metabolism in the placentas of nonsmoking individuals.

Lipoxygenases (LOs), a family of nonheme iron-containing proteins, have a widespread occurrence in plant and animal tissues. Earlier reports demonstrated the presence of LO in the homogenates of rabbit (19) and human placentas (20–22). We have confirmed these reports by purifying the human term placental LO (HTPLO) from nonsmokers by affinity chromatography (23). Although much is known about the pathophysiological role of arachidonic acid metabolites generated via the LO pathway, the significance of co-oxidase activity of LO in the toxicity of chemicals has not yet been fully

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explored. Our laboratory has demonstrated that besides many other xenobiotics, soybean LO (SLO) is capable of oxidizing benzo-(a)pyrene-7,8-dihydriodiol (24) and 2-aminofluorene (25), which are known to cause developmental toxicity or transplacental carcinogenicity in mammals. Joseph et al. (23) observed that similar to SLO, the purified HTPLO can readily metabolize several model substrates including benzidine and its congeners. The purified enzyme was also found to catalyze the co-oxidation of developmental toxicants such as benzo(a)pyrene-7,8-dihydriodiol (26), aflatoxin B₁ (27), and all-trans retinol acetate (28). In the light of these documented results, a consideration of the involvement of HTPLO in 4-ABP metabolism appears worthwhile. The evidence presented here clearly suggests that 4-ABP serves as an excellent substrate for the co-oxidase activity of LO from both the sources.

**Materials and Methods**

**Chemicals.** SLO (Type V), HRP (Type I), LA, γ-linolenic acid, 4-ABP, nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), gossypol and concanavalin-A sepharose 4B (Con-A) were purchased from Sigma Chemical Co. (St. Louis, MO), 5,8,11-eicosatriynoic acid (ETI) and arachidonic acid were procured from Cayman Chemical Co. (Ann Arbor, MI). All other reagents of the highest purity were obtained commercially.

**Enzyme Purification.** Full term placentas (wt. = 500–700 g; N = 10) of nonsmoker women with uncomplicated pregnancies were obtained within 1 hr after cesarean delivery from a local hospital. The smoking status of the mother was determined based on the detailed clinical data of placenta donors. The use of human placentas for this study was approved by the Institutional Review Board, University of South Florida. The processing of chorionic villous tissue samples and the purification of HTPLO by affinity chromatography using a...
Con-A column was carried out as described earlier (23). Only the preparations of HTPLO purified by affinity chromatography were used in all the experiments. The dioxygenase activity of HTPLO towards LA was assayed spectrophotometrically by the detection of 13-hydroperoxy LA which was measured at 234 nm as described previously (23). The dioxygenase activity of HTPLO preparations used in this study ranged from 170 to 225 nmoles/min/mg protein, which is in line with the results reported earlier (23, 27, 28). The protein content was determined by the method of Bradford (29).

**Instrumentation.** A SLM-Aminco DW-2000 UV/Vis spectrophotometer in the split beam mode was used to record difference spectra. Reversed-phase HPLC analysis was conducted using Perkin-Elmer Series 410 LC pumps, Sec-4 solvent controller, Rheodyne 7125 injection system, and a C<sub>18</sub> Bondapak column (3.9 mm I.D. × 30 cm, Waters). HPLC effluent was monitored with a Perkin-Elmer Series LC-235 diode array detector. The mass spectral analysis was performed using a Varian Saturn II gas chromatography-mass spectrometry (GC-MS) equipped with a DB-5 capillary column (0.25 mm I.D. × 30 m, 0.25 μm stationary phase, J & W).

**Spectral Studies of 4-ABP Oxidation.** The reaction medium (final volume 1 ml) contained indicated concentrations of desired polyunsaturated fatty acid, 4-ABP in 10 μl ethanol, and a suitable amount of desired enzyme in 50 mM Tris buffer adjusted at either pH 8.5 (SLO) or pH 7.4 (HTPLO) at 37°C. In each experiment, the reference cuvette containing identical components, except the enzyme, served as the control. The reaction was initiated by the addition of polyunsaturated fatty acid. In some experiments, H<sub>2</sub>O<sub>2</sub> was substituted for polyunsaturated fatty acid. 4-ABP co-oxidation was calculated from the rate of decrease in the absorbance at 270 nm due to 4-ABP depletion. An extinction coefficient (270 nm) of 19.45 mM<sup>-1</sup>cm<sup>-1</sup> for 4-ABP was determined in 50 mM Tris buffer, pH 8.5 or 7.4 and used to calculate the specific activity. The optical difference spectra of those incubations containing either SLO or HTPLO and 4-ABP, obtained after the addition of either LA or H<sub>2</sub>O<sub>2</sub>, were compared with that obtained during 4-ABP (50 μM) oxidation catalyzed by HRP (5 μg/ml) in the presence of H<sub>2</sub>O<sub>2</sub> (100 μM) as described by Hughes et al. (14).

The inhibition of SLO/HTPLO catalyzed 4-ABP depletion was studied under identical experimental conditions as described above in the presence of indicated concentrations of different LO inhibitors. The desired enzyme was preincubated with the specified inhibitor for 3 min in these experiments. In some experiments, either GSH or ascorbic acid was added (1 mM each) to the incubation medium to study their effects on the metabolite formation at 315 nm during SLO-catalyzed 4-ABP co-oxidation. SLO was preincubated with GSH or ascorbic acid for 3 min before the initiation of the reaction with LA, whereas in some experiments GSH or ascorbic acid was added during 4-ABP co-oxidation.

**Fig. 2.** 4-ABP co-oxidation catalyzed by SLO and HTPLO.

The rate of co-oxidation was monitored by measuring the decrease in absorbance at 270 nm due to the depletion of 4-ABP (50 μM) mediated by either (—) 75 nM SLO or (-----) 100 μg/ml HTPLO in the presence of 2 mM LA.

**Fig. 3.** 4-ABP co-oxidation by SLO. Effect of (A) pH, (B) protein, (C) LA and (D) 4-ABP concentration on SLO catalyzed 4-ABP co-oxidation.

See Materials and Methods for additional details.
oxidation. Each experiment was repeated at least three times using different enzyme preparations, and the results are presented as mean ± SEM using descriptive analysis method on Statworks (Macintosh SE). The data were analyzed by ANOVA followed by Fisher’s LSD test and the values with \( p < 0.05 \) were considered significant.

Reversed Phase HPLC Studies for Lipoxygenase Mediated 4-ABP Oxidation. The incubation mixture (final volume 1 ml) contained either SLO (8 \( \mu \)g/ml or 75 nM) or HTPLO (200 \( \mu \)g/ml) and 4-ABP (50 \( \mu \)M) in 50 mM Tris buffer, pH 8.5 or 7.4, respectively. The reaction was initiated by the addition of LA (2 mM). In some experiments, the SLO mediated reaction in 50 mM citrate buffer, was initiated by the addition of \( \text{H}_2\text{O}_2 \) (1 mM). After 5 min of incubation at 37°C, the reaction media were extracted (4 times, 1 ml each) with ice-cold water-saturated ethyl acetate/ether (1:1). The organic phases were pooled, taken to dryness under nitrogen and reconstituted in 300 \( \mu \)l methanol. Aliquots (100 \( \mu \)l) were then analyzed by reversed phase HPLC on the C18 Bondapak column. Solvent A was 10 mM sodium citrate, adjusted to pH 6 with 10 mM citric acid and solvent B was methanol. The column was eluted for 5 min with a 1:1 mixture of A and B followed by a linear gradient to 100% B for 20 min. This was held for 10 min and followed by a return to the initial conditions. The flow rate was maintained at 2 ml/min throughout the operation. The elution profile and retention time of the reaction product generated by either SLO or HTPLO were compared with that of the metabolite produced during the HRP catalyzed 4-ABP oxidation in the presence of \( \text{H}_2\text{O}_2 \). The absorbance of eluate from the reversed phase HPLC column was monitored at 360 nm.

Mass Spectral Analysis of Reversed Phase HPLC Eluate. The fractions of reversed phase HPLC eluate containing a major peak (retention time = 24.5 min) were pooled and extracted (4 times, 1 ml each) with hexane (GC grade). Pooled extracts were then evaporated to dryness under nitrogen and reconstituted in 50 \( \mu \)l hexane. Aliquots (1–10 \( \mu \)l) were analyzed via GC with an ion-trap MS (electron impact scan mode, 1 scan/sec) to obtain a mass spectrum.

Results

Spectral Characterization of 4-ABP Co-oxidation. Co-oxidation of 4-ABP catalyzed by SLO, HTPLO, or HRP was monitored by recording the optical difference spectra in the UV/VIS region. The
Results are similar to and confirm those reported by Hughes et al. (14). The optical difference spectra of incubation media containing 4-ABP by HRP are shown in fig. 1. A concurrent absorbance increase within a wavelength range of 300–400 nm suggested the formation of 4-ABP metabolite(s). As shown in fig. 1B, a gradual increase in the absorbance with the formation of peak at 325 nm was observed in the reaction mixture containing H2O2 and SLO. When H2O2 was replaced by LA, the metabolite peak shifted towards lower wavelength and the absorbance maximum was noted at 315 nm (fig. 1C). It is believed that this peak shift is a result of the presence of LA metabolite(s) in the reaction media. Similar to SLO, affinity purified HTPLd was also found to catalyze 4-ABP co-oxidation in the presence of LA as shown by similar changes in the optical difference spectra including the appearance of a peak at 315 nm (fig. 1D).

### Effects of Different Experimental Conditions on 4-ABP Co-oxidation

The SLO catalyzed 4-ABP depletion in the presence of LA was examined under a variety of experimental conditions. The linear portion of recording of absorbance loss at 270 nm reflecting depletion of the substrate (fig. 2) was used to calculate the rate of enzymatic 4-ABP co-oxidation. Fig. 3A shows the effect of pH on the rate of 4-ABP depletion. A gradual increase in the reaction rate was observed with an increase in pH of the medium. Maximum specific activity was noted at pH 8.5. A further increase in pH resulted in a decline in reaction velocity. In view of this, all the subsequent LA-supported co-oxidation assays with SLO were performed in 50 mM Tris buffer, pH 8.5. The amount of 4-ABP disappeared was the function of SLO concentration employed in the assay medium (fig. 3B) and was linear up to 100 pmoles of the enzyme/ml. Boiled (100°C for 5 min) enzyme preparation did not cause any time dependent 4-ABP depletion. The specific activity of the enzyme exhibited an increase with an increase concentration (fig. 3C) and reached maximum at 2 mM LA. Further increase in the LA concentration (fig. 3C) and reached maximum at 2 mM LA. Under optimum conditions, a rate of 350 ± 41 nmoles of 4-ABP depleted/min/nmole of enzyme (SLO) was noted in various experiments, when incubations were performed in the presence of LA. 4-ABP depletion also showed a dependency on the substrate concentration (fig. 3D). The Lineweaver-Burk plot of the data yielded an apparent K_m value of 9.5 μM for 4-ABP and a V_max value of 403 nmoles of 4-ABP depleted/min/nmole of SLO.

Using an experimental approach similar to that employed for the SLO, the 4-ABP depletion catalyzed by the affinity purified HTPLd was characterized. As shown in fig. 4A, the depletion of 4-ABP increased linearly with an increase in the enzyme concentration (up to about 120 μg/ml). The presence of 2 mM LA was required to observe maximum specific activity (fig. 4B). Further increase in the LA concentration did not change the rate of 4-ABP depletion. The reaction also exhibited dependency on 4-ABP concentration (fig. 4C).

Under the experimental assay conditions employed, different preparations of HTPLd yielded an average specific activity of 45 ± 6 nmoles of 4-ABP depleted/min/mg protein. (N = 4). The analysis of the data using Lineweaver-Burk plot yielded a K_m value of 10.8 μM for 4-ABP and a V_max value of 50.8 nmoles of 4-ABP depleted/min/mg protein. The control experiments carried out in the absence of either enzyme or LA or in the presence of heat denatured HTPLd showed no significant amount of 4-ABP depletion.

### Table 2

**Ability of different polyunsaturated fatty acids in supporting 4-ABP co-oxidation catalyzed by SLO and HTPLd**

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Final Conc. (mM)</th>
<th>Rate of 4-ABP Co-oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SLO</td>
<td>HTPLd</td>
</tr>
<tr>
<td></td>
<td>Specific Activity</td>
<td>Relative Activity</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>1</td>
<td>303.4 ± 28.5†</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>1</td>
<td>75.7 ± 10.8</td>
</tr>
<tr>
<td>γ-Linolenic acid</td>
<td>1</td>
<td>22.6 ± 2.0</td>
</tr>
</tbody>
</table>

* Specific activity is expressed as nmoles of 4-ABP depleted/min/nmole of enzyme. Values indicated are mean ± SEM (N = 4).
** Specific activity is expressed as nmoles of 4-ABP depleted/min/mg protein. Values indicated are mean ± SEM (N = 4).
† Significantly greater than other specific activities given in the same column (p < 0.05), based on ANOVA followed by Fisher’s LSD test.

### Table 3

**Inhibition of 4-ABP co-oxidation catalyzed by SLO and HTPLd**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final Conc. (μM)</th>
<th>Rate of 4-ABP Co-oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SLO, Relative Activity</td>
<td>HTPLd, Relative Activity</td>
</tr>
<tr>
<td>NDGA</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>Gossypol</td>
<td>1</td>
<td>45 ± 6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>ETI</td>
<td>1</td>
<td>64 ± 8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>BHT</td>
<td>1</td>
<td>61 ± 4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>BHA</td>
<td>1</td>
<td>64 ± 6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>46 ± 4</td>
</tr>
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<td></td>
<td>10</td>
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</tbody>
</table>

Values indicated are mean ± SEM (N = 4). Control specific activities (shown as 100 relative activity) towards 4-ABP for SLO and HTPLd were 350 ± 41 nmoles of 4-ABP depleted/min/nmole of SLO and 45 ± 6 nmoles of 4-ABP depleted/min/mg protein, respectively.

Reaction mixture (final volume 1 ml) contained 75 pmoles of SLO or 80 μg HTPLd, 50 μM 4-ABP, 2 mM linoleic acid, and indicated concentration of inhibitors in 50 mM Tris buffer, pH 8.5 and pH 7.4, respectively.

All values observed in the presence of inhibitors tested are significantly less than the respective control (p < 0.05), based on ANOVA followed by Fisher’s LSD test.
A comparison of the specific activity on the basis of per mg protein suggested that SLO in the presence of LA is about 70 fold more efficient than either SLO with H₂O₂ or HTPLO with LA in co-oxidizing 4-ABP (table 1). However, HTPLO in the presence of LA appeared to be almost 2-fold more efficient in catalyzing 4-ABP co-oxidation than SLO (180 vs 93 nmoles/min/unit of dioxygenase activity, respectively) when the comparison of specific activities was expressed per unit of dioxygenase activity of respective enzyme.

**Effect of Different Polyunsaturated Fatty Acids on 4-ABP Co-oxidation.** Besides LA, other polyunsaturated fatty acids such as γ-linolenic acid and arachidonic acid also supported the SLO and HTPLO mediated 4-ABP co-oxidation. Although indepth optimization studies were not performed, when tested at the equimolar concentration (1 mM), LA was most effective in supporting 4-ABP co-oxidation, while γ-linolenic acid and arachidonic acid were less than half as efficient with both the LOs (table 2).

**Inhibition of Lipoxygenase catalyzed 4-ABP Co-oxidation.** Several inhibitors of dioxygenase activity of LO were tested as potential inhibitors of the enzyme mediated 4-ABP co-oxidation. Table 3 shows the concentration dependent inhibition of SLO catalyzed 4-ABP depletion by different inhibitors in the presence of LA. Except for ETI, five micromolar or lower concentration of these inhibitors was required to observe a significant inhibition of the reaction. Among the inhibitors tested, ETI was the least effective and required relatively higher concentration (10 µM) to observe about 50% inhibition of the SLO catalyzed 4-ABP co-oxidation.

All the known LO inhibitors tested also caused a significant inhibition of the reaction mediated by HTPLO. NDGA and gossypol (5 µM each), caused about 65–70% inhibition of this enzymatic reaction. BHT, BHA, and ETI (10 µM each) blocked the HTPLO catalyzed 4-ABP depletion by about 50% (table 3). Taken together (figs. 1–4; tables 1–3), these data suggest the enzymatic nature of 4-ABP depletion by SLO and HTPLO.
The ability of GSH and ascorbic acid to suppress the stable metabolite formation during SLO catalyzed 4-ABP co-oxidation in the presence of LA was monitored spectrally. Both the rate of change in absorbance and the absolute absorbance measured at 315 nm as a result of the accumulation of a stable metabolite decreased upon preincubation of SLO and 4-ABP with 1 mM GSH compared with the control (fig. 5A). An addition of 1 mM GSH after the initiation of reaction at the indicated time also decreased the 4-ABP metabolite formation (fig. 5C). Similarly, 1 mM ascorbic acid completely blocked both the rate of reaction and the absolute absorbance measured at 315 nm as a result of 4-ABP metabolite generated in the presence of SLO (fig. 6A-C). These observations indicate the generation of 4-ABP free radical as the initial oxidation product and its reduction back to 4-ABP during the process of co-oxidation by SLO.

Characterization of 4-ABP metabolite. The stable metabolite of 4-ABP oxidation accumulating in the reaction media containing HRP or LOs was isolated by reversed phase HPLC technique as described in Materials and Methods. HPLC profiles indicated the presence of a single metabolite eluting at 24.5 min in the organic extract of the HRP-containing incubation media supplemented with H₂O₂ (fig. 7A). Similar results were observed with SLO when the reaction media were supplemented with either H₂O₂ (fig. 7B) or LA (fig. 7C). The LA-dependent HTPLO catalyzed reaction also yielded a single metabolite exhibiting the same retention time on the HPLC column (fig. 7D). These results suggest that only one identical metabolite is generated by HRP, SLO, and HTPLO. The elution of the metabolite from the HPLC column was monitored at 360 nm. This is based on the expected presence of an azo linkage in the molecule. It is important to mention that azo double bond as a chromophoric group absorbs within a range of 350–370 nm (30).

The overall chemical structure of 4,4'-azobis(biphenyl) as the final stable metabolite was confirmed by the mass spectroscopic study (fig. 8A-D). As expected, the molecular ion was observed in each case at m/z 335. This may be a result of the addition of one hydrogen ion to 4,4'-azobis(biphenyl) (mol wt = 334) forming the molecular ion (m/z 335) observed. The cleavage of the nitrogen phenyl bond produced most stable daughter ion at m/z 153 along with minute fragment at m/z 181. Azo double bond upon cleavage produced fragments at m/z 167. From this additional chemical analysis, 4,4'-azobis(biphenyl) would be the only expected metabolite formed upon coupling of two 4-ABP free radicals initially produced during the HRP as well as SLO and HTPLO catalyzed co-oxidation of 4-ABP.

Discussion

4-ABP and related arylamines represent an important group of chemicals that deserve some attention since the possibility exists for...
Co-oxidation of 4-Aminobiphenyl by Lipooxygenase

Fig. 9. Proposed mechanism of lipooxygenase catalyzed 4-ABP co-oxidation.

Exposure of pregnant women to these chemicals through environmental, occupational, and/or dietary sources. Among different arylamines, 4-ABP, a potent carcinogen, occurs in the environmental tobacco smoke to which nonsmokers may also be exposed. Because the emissions of 4-ABP are much higher (30-fold) in the sidestream smoke than in the mainstream smoke (2), passive smokers might receive substantial doses of this carcinogen. Transplacental transfer of 4-ABP both in human (6) and laboratory animals (10) has already been reported. These findings are further supported by the detection of the DNA (8) and hemoglobin (9) adducts of 4-ABP or its metabolites in the placentas of nonsmokers. Several studies (4–6) have suggested that 4-ABP may be a transplacental carcinogen in man.

The studies with laboratory animal species have established that the metabolism of parent arylamines generates reactive electrophilic intermediates that bind to vital macromolecules like DNA and/or protein, and this is believed to be the mechanism responsible for the initial toxic insult leading to the carcinogenic effects (31). Since N-oxidation of the parent arylamine is generally regarded as the critical step in the metabolic activation of 4-ABP to a proximate carcinogen, the formation of 4-ABP-DNA and 4-ABP-hemoglobin adducts was considered as an index of in vivo or in vitro N-oxidation by the mammalian hepatic cytochrome P-450 (11, 12). Besides cytochrome P-450, prostaglandin synthase from the ram seminal vesicles has also been found to be capable of mediating 4-ABP co-oxidation and concomitant covalent binding of its oxidized metabolite to DNA, r-RNA or protein (13, 32). In addition, Hughes et al. (14) have reported 4-ABP as a reducing co-substrate for HRP and is oxidized to a stable azo compound [4,4′-azo(biphenyl)] through a reaction mechanism involving initial free radical formation.

The presence of microsomal cytochrome P-450 (16) capable of xenobiotic oxidation and prostaglandin synthase (15) are almost undetectable in the human term placenta if the donor is a nonsmoker. In contrast, LO occurs in abundant quantities in the human term placentas of nonsmokers (23). The affinity purified enzyme was found to readily oxidize a wide range of chemicals including benzidine, an arylamine structurally related to 4-ABP (26–28). To our knowledge, no published information is available regarding any enzyme system responsible for 4-ABP metabolism in the human term placenta. In view of this, it was of interest to evaluate whether 4-ABP can serve as a substrate for the HTPLO. For comparison, similar studies were carried out with SLO, a model enzyme widely used to study the LO-mediated xenobiotic oxidation, while HRP was used as a positive control.

Co-oxidation of 4-ABP by both the LOs was examined by monitoring the change in the UV/VIS absorbance spectrum of 4-ABP. A rapid decrease in the absorbance at 270 nm, the absorption maximum of 4-ABP, was noted after the addition of LA/H2O2 to the reaction media containing either HRP, SLO, or HTPLO. A concomitant increase in the absorbance within a range of 300–400 nm (fig. 1) was also observed. These spectral shifts, observed only after the addition of LA/H2O2, suggested that similar to HRP, 4-ABP is co-oxidized by both SLO and HTPLO. The oxidation of another arylamine, benzidine, by the HRP and human liver LO (33) also results in similar changes in the UV/VIS absorption spectrum. 4-ABP co-oxidation catalyzed by SLO/HTPLO was dependent upon the concentration of protein, LA, and 4-ABP in the incubation medium (figs. 3 and 4). No significant 4-ABP co-oxidation occurred when boiled enzyme was used. A marked inhibition of 4-ABP co-oxidation by the classical LO inhibitors (table 3) strongly suggests the involvement of LO and an enzymatic nature of this reaction. The blockade of the reaction by BHA and BHT is indicative of the participation of free radicals in the LO mediated 4-ABP co-oxidation.

The inhibition of the new peak formation at 315 nm by GSH and ascorbic acid (figs. 5 and 6) provides additional evidence that a free radical metabolite of 4-ABP was formed by LO during the reaction. Although a detailed study of the nature of the inhibition involved was not carried out, both GSH and ascorbic acid are expected to nonenzymatically reduce the free radical metabolite back to 4-ABP. GSH (34) and ascorbic acid (35) have also been shown to be oxidized by LO and, thus they may suppress 4-ABP oxidation by acting as the competitive substrates. Another possibility for the decrease in absorbance at 315 nm in the presence of GSH is the formation of a conjugate resulting from the reaction of GSH with the 4-ABP free radical metabolite. Similar GSH conjugate formation has previously been reported for benzidine (36). Further study is needed to reveal the mechanism involved in the observed inhibition of 4-ABP co-oxidation.

The detection of a major metabolite peak exhibiting a retention time of 24.5 min following reversed phase HPLC (fig. 7) suggests the generation of a stable 4-ABP metabolite following its co-oxidation by SLO/HTPLO, and this metabolite was identified by GC-MS as 4,4′-azobis(biphenyl). The mass spectral analysis (fig. 8) firmly implicates the formation of 4-ABP free radical as the initial metabolite that dimerizes to a coupling azo product. This contention is further supported by similar observations regarding the generation of a benzidine free radical catalyzed by peroxidases (37, 38) and subsequent coupling of two benzidine free radicals to yield azobenzidine (39).

The generation of free radicals during LO catalyzed dioxygenation of polynsaturated fatty acids is well established. One of the major free radical species generated is the oxygen derived lipid peroxyl free radical (LOO•). It is believed that the LOO• causes one electron oxidation of 4-ABP, thereby generating 4-ABP free radical, the corresponding oxidized species (fig. 9). Similar to HRP, peroxidase-like activity of LO was noted during 4-ABP oxidation in the presence of H2O2 (fig. 1B, table 1). However, the specific activity observed in the presence of LA was much higher (73-fold) as compared with that noted in the H2O2-supplemented reaction media. Since co-oxidation of 4-ABP is primarily driven by the oxidant (LOO•) generated during dioxygenase reaction, it is more meaningful to express co-oxidase specific activity on the basis of units of dioxygenase activity. Interestingly, HTPLO seems to be far more efficient (2-fold) than SLO.
when data are expressed as per unit of dioxygenase activity (table 1). These results strongly suggest a definitive role for LO catalyzed 4-ABP co-oxidation in the human term placentas of nonsmokers. Whether human intrauterine conceptual tissues including embryo/fetus can mediate a similar co-oxidation is unknown. However, considering the remarkable similarity in the catalytic activities of LO isolated from the human term placentas and human intrauterine conceptual tissues during the crucial organogenesis period (2–8 weeks of gestation) in the co-oxidation of transplacental toxicants such as benzo(a)pyrene-7,8-dihydrodiol (26), aflatoxin B1 (27) and carcinogenic arylamine like benzidine (40), a significant amount of LO mediated 4-ABP 7,8-dihydrodiol (26), aflatoxin B1 (27) and carcinogenic arylamine (111) co-oxidation is unknown. However, considering the human term placentas and human intrauterine conceptual tissues can mediate a similar co-oxidation is unknown. Whether human intrauterine conceptual tissues including embryo/fetus can mediate a similar co-oxidation is unknown.

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